Developmental Cell, Vol. 3, 615–623, November, 2002, Copyright ©2002 by Cell Press

Morphogen Transport along Epithelia, an Integrated Trafficking Problem

Review

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Graded signals are an important component of current models of pattern formation. Typically, a group of cells produces a signal that decays as it spreads through neighboring tissue. By contrast with endocrine signals, which spread systemically, patterning signals or morphogens have a restricted zone of influence, an area classically known as a field. The widely accepted model is that graded distribution of such signals allow cells to measure their position relative to the source. Although it provides a framework for understanding pattern formation, the concept of the morphogen raises many mechanistic issues. For example, how the distribution of a morphogen is established and maintained remains an outstanding issue. There is no doubt that signals are transported over distances of tens of cell diameters and that stable gradients do form. The question of how this is achieved has aroused the interest of many cell biologically minded developmental biologists.

Two Models of Transport

Three broadly defined processes can affect the range and slope of a morphogen gradient: the rate of production from the source, the rate of transport, and the rate of degradation. Among these three processes, transport has recently attracted considerable attention. Note that the word transport is used here in a mechanistically neutral way (as in electrophysiology or solid-state physics), and simply refers to the displacement of molecules. Two types of mechanisms are currently being considered. One model is that transport occurs by diffusion (Crick, 1970). However, many feel that an important process such as the generation of a morphogen gradient could not be left to the vagaries of passive diffusion and thus believe that active mechanisms of transport are at work. In particular, the model of planar transcytosis (Gonzalez et al., 1991; Bejsovec and Wieschaus, 1995) has recently gained experimental support (Entchev et al., 2000). According to this model, receiving cells internalize the ligand and then recycle it to the cell surface, thus presenting it to further cells and allowing its spread along the tissue. Formally, if recycling is not spatially directed (e.g., along a specific embryonic axis), transcytosis could be represented by a diffusion term ($D\ \delta^2 L/\delta x^2$) in a transport equation, as it would follow a random walk behavior. However, one important difference between the two modes of transport is that one is active while the other is passive. Another difference between the two models relates to the route followed by the signal. One is strictly extracellular, while the other requires vesicular trafficking within cells. Although these are important differences, experimental distinction between the two models has turned out to be very difficult, in part because transport cannot be considered in isolation from ligand production and degradation. For example, even if diffusion is the main contributor to transport, this could be obscured by a role of endocytosis in degradation. In this review, we consider the various characteristics that a transport mechanism must fulfill and how various cell biological parameters affect such requirements. When appropriate, the predictions made by both models will be contrasted. Additional models have been suggested, but we will only consider them in passing.

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Experimental Systems

Pattern formation often occurs in epithelial sheets. However, signals have also been shown to pattern three-dimensional structures. Thus, depending on tissue organization, a given signal is expected to spread in two or three dimensions. For example, a TGFβ produced from the Niewkoop center in the *Xenopus* blastula is believed to spread throughout the vegetal hemisphere and organize the dorsoventral axis (Green et al., 1992; Gurdon and Bourillot, 2001). The endogenous gradient has not been visualized, but radiolabeled Activin has been shown to spread from soaked beads into surrounding blastula cells and form a detectable gradient over 100 μm in about 1 hr (Dyson and Gurdon, 1998). Another three-dimensional field is the limb bud, where Sonic Hedgehog (Shh) produced from the zone of polarizing activity (ZPA) is presumed to spread through mesenchyme and specify different digits along the anterior-posterior axis (Lewis et al., 2001). Although in these two examples pattern formation clearly occurs within a mass of cells, in many other instances signals organize patterns in epithelia. For example, in vertebrate embryos, such as chicks, Shh produced from the notochord and ventral neural tube specifies distinct fates along the surface of the neural tube (Briscoe et al., 2001). Additional examples come from *Drosophila*, which has so far been a model of choice for studies of signal transport along epithelia. Therefore, work with *Drosophila* forms the basis of much of this review.

Two main epithelia of *Drosophila* are the subject of intense investigation, the embryonic epidermis and imaginal disks. Embryonic development occurs relatively quickly and with a relatively small number of cells. For example, at least four signals operate within each segment, at a time when the segments themselves are barely 11 cells wide (Alexandre et al., 1999). For these reasons, the embryonic epidermis of *Drosophila* may not be ideal to study long-range transport. By contrast, in wing imaginal disks (see diagram in Figure 1), two

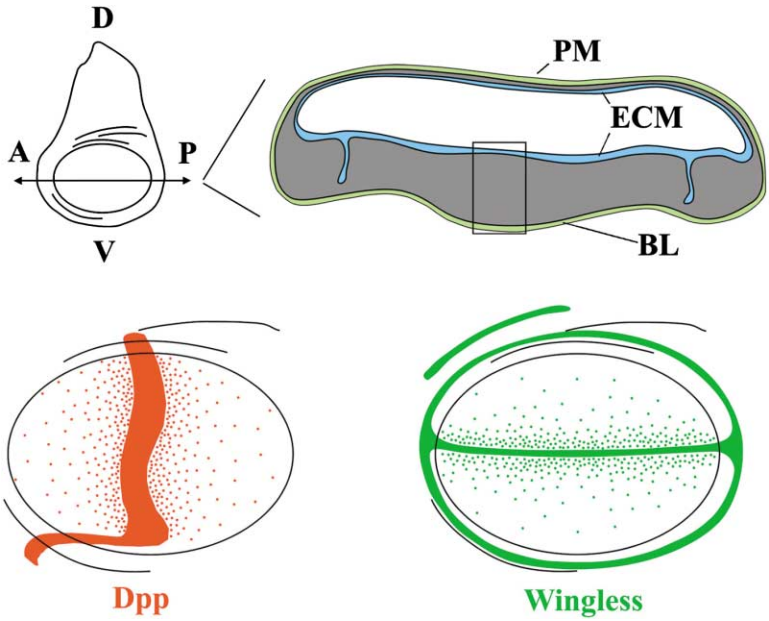

Figure 1. General Architecture of Wing Imaginal Disks

Disks are folded epithelial structures composed of the epithelium proper and the overlying peripodial membrane (labeled PM in the cross-section). Because of this topography, the apical surface, which is covered by extracellular matrix (blue shading), faces the inside of the disk and is therefore not readily accessible to externally applied agents. ECM refers to the extracellular matrix and BL, the basal lamina. Only the central part of the epithelium proper (the pouch) gives rise to the wing. Two main signals operate in the pouch. Wingless (shown in green) is expressed at the dorsoventral boundary and Dpp (red) is expressed along the boundary separating the anterior and posterior compartments. Expression of Wingless around the pouch does not contribute to dorsoventral patterning and is not considered here. The box within the section is enlarged in Figure 2 with the same color coding.

signals are known to act at a relatively long range (as shown by direct visualization and target gene expression). Wingless spreads from the dorsoventral boundary and act over a range of up to 25 cell diameters (Zecca et al., 1996; Strigini and Cohen, 2000), while Dpp, a BMP homolog, spreads over a similar range in the orthogonal direction, from the anterior-posterior boundary (Nellen et al., 1996; Entchev et al., 2000; Teleman and Cohen, 2000). Hedgehog (Hh) also acts as a morphogen in disks but over a more limited range (Basler and Struhl, 1994; Zecca et al., 1995; Strigini and Cohen, 1997). Although less accessible than the embryonic epidermis, imaginal epithelia can be imaged live, thus allowing GFP fusion proteins to be followed in real time. Imaging and interpretation of fixed preparation is also facilitated by the fact that imaginal disks are essentially two-dimensional structures. This advantage, coupled with the power of *Drosophila* genetics, makes imaginal disks an impressive system to study signal transport. It is thought that such studies will be relevant to a wide variety of systems. However, because of their two-dimensional configuration, studies with imaginal disks may miss aspects that are specific to a three-dimensional configuration.

Imaging Gradients

In the past, morphogens have been difficult to visualize, but recent developments have allowed two of the best-studied morphogens (Dpp and Wingless) to be imaged both with antibodies and GFP fusion proteins (Entchev et al., 2000; Strigini and Cohen, 2000; Teleman and Cohen, 2000; Pfeiffer et al., 2002; Srinivasan et al., 2002). The most evident feature is that staining is punctate, presumably reflecting continuous endocytosis. Importantly, the number of punctae decreases with distance from the source, as expected from a morphogen. In addition to punctae, diffuse staining is also seen. Within the limitation of low-level fluorescence imaging, optical sections suggest that diffuse Dpp is mostly present around the basolateral surface of cells (except at the source; see below). To investigate whether diffuse Dpp is present on the inside or the outside of cells, imaginal disks were stained with antibodies prior to fixation (hence permeabilization), thus enabling access only to the extracellular space. Subsequent detection with secondary antibodies showed that Dpp is present extracellularly and that its distribution there is also graded (Teleman and Cohen, 2000). Note that as this procedure only exposes the basal surface of the disk to the staining solution (see Figures 1 and 2), it confirms that extracellular ligand is present on the basolateral surface but does not exclude an apical presence. In a second approach, intact disks were treated with proteinase K to specifically digest extracellular proteins (Teleman and Cohen, 2000). The majority of mature Dpp was digested, suggesting that it is exclusively present within the basolateral extracellular space (although further controls are needed to prove that proteinase K does not interfere with the integrity of the epithelium). Wingless too is believed to localize on the basolateral surface of receiving disk cells (Strigini and Cohen, 2000). Note that both Dpp and Wingless are thought to be secreted on the apical surface. In the case of Dpp, this is suggested by its apical localization in secreting cells (Entchev et al., 2000; Teleman and Cohen, 2000). For Wingless, at least in the embryo, the evidence is stronger (Simmonds et al., 2001), and indeed, secretion of GFP-Wingless has been directly observed on the apical side (Pfeiffer et al., 2002). If ligands are indeed secreted apically, it will be important to find out how they make their way to the basolateral space of the epithelium (see Figure 2). In any case, the presence of ligand both within and outside cells is compatible with both models of transport.

Basic Requirements: Transport Must Be Nondirectional and Rapid

In imaginal disks, clones of cells that ectopically express either Wingless or Dpp activate downstream targets symmetrically in all directions (Lecuit et al., 1996; Nellen

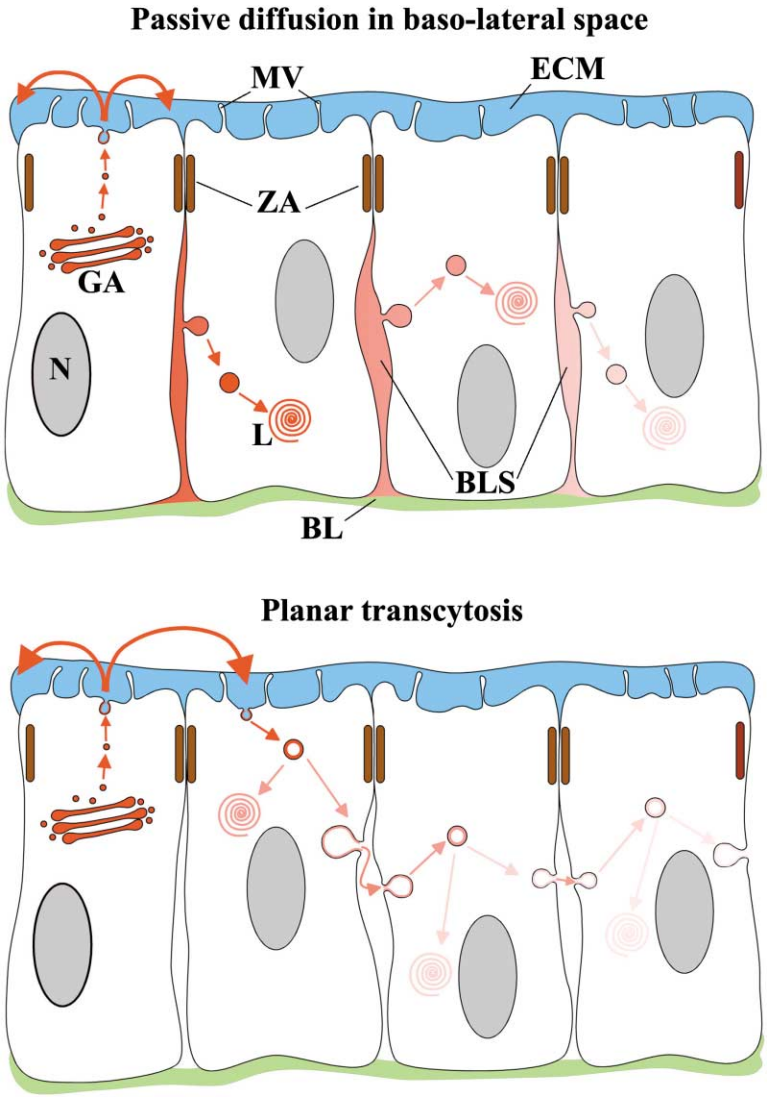

Figure 2. Two Main Models of Transport

Examination of wing disks at high magnification reveals the following structures: microvilli (MV), zonulae adherens (ZA), and a basal lamina (BL). The basolateral membrane (the portion of membrane located more basally than the ZA) of adjoining cells is not closely apposed. This creates an interstitial space (basolateral space, BLS) where ligand might diffuse. In both models, the Golgi apparatus (GA) and the lysosomes (L, represented by a coil) are colored in red. Morphogens such as Wingless and Dpp are thought to be secreted at the apical surface (shown with red arrows). It is not clear how the ligand crosses the epithelial barrier from apical to basal, a problem that is particularly acute for the diffusion model.

Passive diffusion in basolateral space: in this model, following secretion, the ligand diffuses freely in the extracellular basolateral space. Degradation of the ligand is expected to occur mostly by targeting to lysosomes (L).

Planar transcytosis: in this model, ligand is transported along the plane of the epithelium by repeated cycles of endocytosis and recycling to the cell surface. In order for a gradient to form, at each cycle a subset of internalized ligand is targeted to lysosomes for degradation and the relative rates of recycling and degradation would specify the slope of the gradient. In the diagram, endocytosis and recycling are shown to occur at the basolateral membrane, but this has not yet been shown experimentally.

et al., 1996). As expected, GFP-Dpp spreads symmetrically around expressing clones (Entchev et al., 2000). This confirms that cells of the epithelium are not programmed to move signals in any given direction and allows the elimination of convective flow as a form of transport. Such a process has recently been suggested to operate in the specification of the left-right axis in vertebrates. There, it is thought that bulk flow of extracellular medium overlying the embryonic node (a recognizable structure that acts as an organizer; Beddington and Robertson, 1999) biases the distribution of a left-right signal (Nonaka et al., 2002). The nondirectionality of morphogen transport makes it unlikely that morphogens are transported by a similar process, at least in *Drosophila* imaginal disks.

Several experimental avenues have shown that morphogen gradients form rapidly. In one experiment, the temperature sensitivity of the Gal4 system was used to activate the production of GFP-Dpp at a specific time, and formation of the gradient was followed in time (Entchev et al., 2000). Expression was activated by shifting disks from 16°C (when Gal4 is less effective) to 25°C (when it is fully active). Although there are technical limitations (Gal4 is still active at 16°C), this experiment showed that Dpp moves at a rate of four cell diameters/hr until a steady state is reached, about 8 hr after the onset of expression. Similar results were obtained using a temperature-sensitive allele of *hh* to control the timing of Dpp production (Teleman and Cohen, 2000). (Hh is a signal required for transcription of Dpp; Basler and Struhl, 1994). After some time at restrictive temperature, the Dpp gradient disappears due to lack of production. Reactivation of production by returning to the permissive temperature leads to rapid formation of a new gradient. For Wingless, a temperature-sensitive allele of *shibire*, the gene encoding Dynamin (Figure 3), was used for temporal control of production (Strigini and Cohen, 2000). Although Dynamin, a GTPase involved in pinching vesicles off the cell membrane, is best known for its general role in endocytosis (Hinshaw, 2000), it is also required (albeit in a variable way) for secretion of Wingless (Strigini and Cohen, 2000). Therefore, at the restrictive temperature, cells at the dorsoventral boundary, which normally secrete Wingless, accumulate Wingless

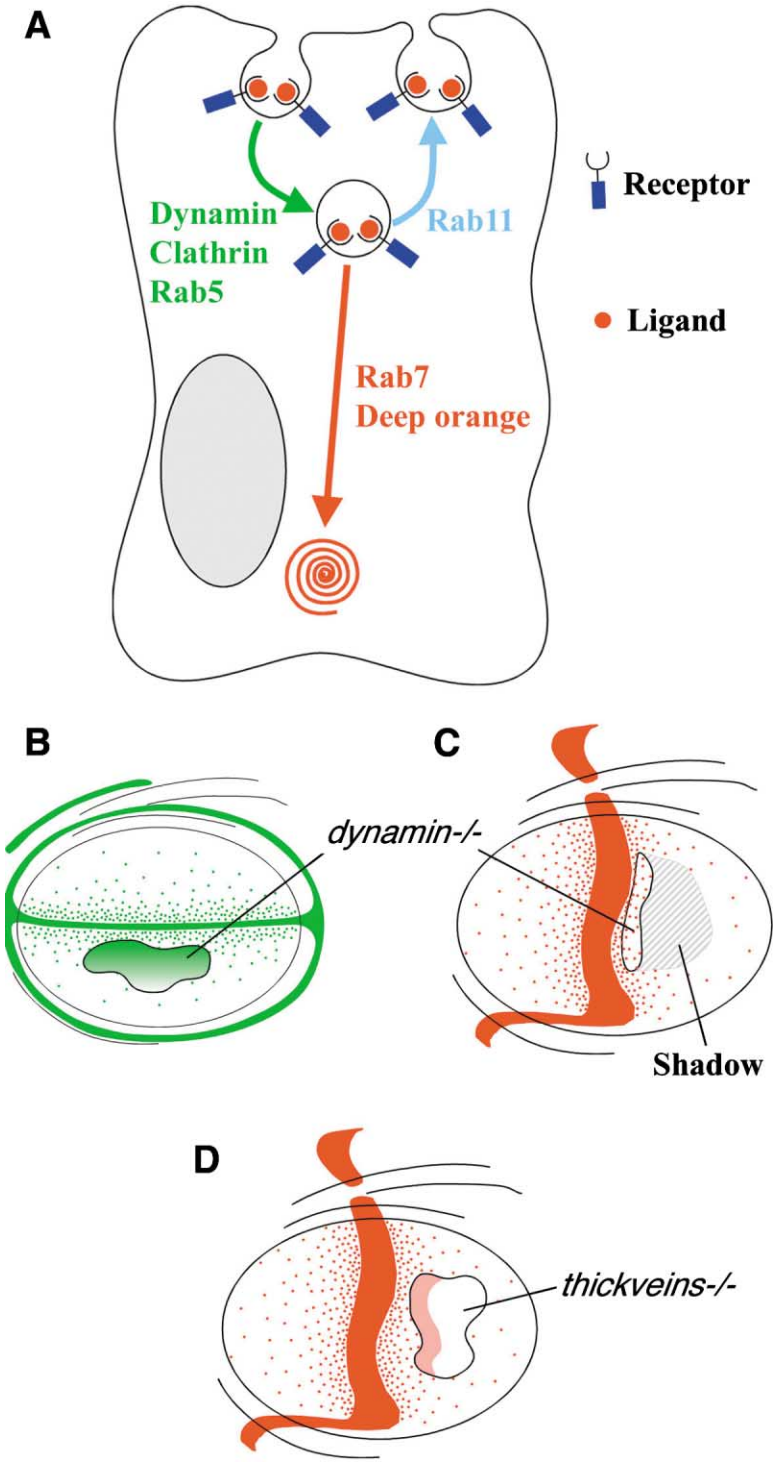

Figure 3. Tools Currently Available to Interfere with Trafficking

(A) Trafficking can be affected by changing the activity of several regulators (reviewed by Seto et al., 2002). Regulators of endocytosis include the small GTPase Rab5, Clathrin, and Dynamin (encoded by *shibire* in *Drosophila*). Recycling from the recycling endosome requires Rab11 (although other means of recycling can also operate). Targeting to late endosomes/lysosomes requires Rab7 (Zerial and McBride, 2001), and also, in *Drosophila*, the gene *deep orange*, which encodes a homolog of the yeast vacuolar protein-sorting protein, Vps18p (Sevrioukov et al., 1999). A way to interfere specifically with trafficking of a specific ligand would be to identify and mutate trafficking signals present in the cytoplasmic tails of receptors (blue box). Such an approach has not yet been followed for developmental receptors.

(B) Effect of *shibire* mutant clones on Wingless distribution: in *shibire* mutant cells, Wingless is no longer internalized and accumulates at the cell surface, but the spread of Wingless is not blocked (Strigini and Cohen, 2000). This suggests that Wingless can spread without endocytosis in the extracellular space, although confirmation awaits the same experiment being performed dynamically.

(C) Effect of *shibire* mutant clones on Dpp distribution: here, the assay was done dynamically (Entchev et al., 2000); hence, endocytic vesicles containing Dpp can still be detected in mutant cells. Under appropriate conditions, these cells cast a shadow (absence of Dpp-containing vesicles) behind them, as if they were unable to pass the ligand to the next cells, thus behaving like a barrier.

(D) Effect of *thickveins* (*tkv*) mutant clones on Dpp movement. Dpp accumulates at the surface of mutant cells situated at the edge of the clone next to the source, and is no longer internalized.

intracellularly. At the same time, the Wingless gradient progressively decays elsewhere in the disk. Return to the permissive temperature releases Wingless from the source and within 1 hr, the gradient is reconstituted, implying a rate of transport of about 50 μm/hr. One shortcoming of this experiment is that loss of Dynamin function is likely to affect many processes. Nevertheless, it provides additional evidence that gradients form rapidly and are continuously maintained. The speed of gradient formation puts a constraint on the kinds of mechanism that can generate gradients, at least in this system. For example, as the average cell doubling time is around 11 hr in imaginal disks (Neufeld et al., 1998), the gradient could not be formed by inheritance of the signal through cell proliferation, at least in general. Proliferation could contribute, but only in specific situations where it is rapid and the range is relatively short such as in the embryonic epidermis of *Drosophila* (Pfeiffer et

al., 2000). Because of its high throughput nature, the imaginal disk gradient is also unlikely to be stably stored in the extracellular matrix. According to a recent mathematical model (Lander et al., 2002), rapid gradient formation is also incompatible with planar transcytosis, because it would require many trafficking processes to occur at unusually high rates. We will return to this issue at the end.

Ligand Internalization and Degradation

One corollary of the high throughput nature of the Dpp and Wingless gradients is that both ligands are expected to turn over rapidly. Indeed, surface-labeled Wingless or GFP-Dpp (with biotin) is rapidly brought to undetectable levels within a 3 hr chase period (Teleman and Cohen, 2000). Degradation could occur either as a result of proteolytic action in the extracellular space or by internalization and targeting to lysosomes. As discussed below, both processes probably occur, although endocytic trafficking is likely to be of prime importance to the shaping of morphogen gradients.

Many ligands are internalized by receptor-mediated endocytosis (Figure 3). Therefore, the punctate distribution of ligand in receiving cells most likely represents an endocytic compartment. Indeed, internalized fluorescent Dextran (from an externally applied solution) colocalizes, at least partially, with intracellular Dpp or Wingless (Entchev et al., 2000; Pfeiffer et al., 2002). The membrane dye FM4-64, another endocytic marker, also colocalizes with Wingless in wing imaginal disks (Greco et al., 2001). To verify that internalization requires Dynamin (Figure 3), clones of *shibire*^{ts} cells were produced using the Flp/FRT system. They were allowed to grow at permissive temperature and then shifted to restrictive temperature and examined 3 hr later. At this time, intracellular Wingless-containing vesicles are no longer detectable in mutant cells (Strigini and Cohen, 2000). Therefore, Wingless is indeed internalized by a Dynamin-dependent process. Analogous experiments showed that no internalized Dpp can be detected in *shi*^{ts} cell clones kept at the restrictive temperature for 6 hr (Entchev et al., 2000).

Endocytosis is an essential step toward degradation in lysosomes. As might be expected, excess Wingless accumulates at the surface of *shibire* mutant cells (Figure 3; Strigini and Cohen, 2000). However nonlysosomal means of degradation are also likely to play a role. When whole *shibire*^{ts} disks are placed at restrictive temperature, Wingless supply is cut off and such disks lose all Wingless immunoreactivity (both intracellular and extracellular) within 3 hr of the shift (Strigini and Cohen, 2000). Thus, when its production is impaired, the ligand is cleared from the epithelium even in the absence of endocytosis. This suggests that an unidentified extracellular protease could contribute to ligand degradation. A precedent for extracellular degradation has been documented for Sog, a secreted inhibitor of Dpp signaling, which forms a ventral-to-dorsal gradient in the embryonic epidermis. Sog has been known for a while to be cleaved by the extracellular metalloprotease encoded by tolloid (Marques et al., 1997). Recent evidence shows that it is also downregulated by Dynamin-dependent endocytic trafficking (Srinivasan et al., 2002).

Even though extracellular proteases probably contribute to signal degradation, current evidence suggests that endocytic trafficking is a key regulatory process. For example, compromising lysosomal degradation of Wingless either genetically (by reducing the activity of *deep orange*; Figure 3) or chemically (with chloroquine, an inhibitor of lysosomal function) leads to increased range of Wingless and excess signaling in the embryonic epidermis (Dubois et al., 2001). Conversely, in imaginal disks, expression of a dominant active Rab7 (a small GTPase required for sorting into late endosomes; see Seto et al., 2002 for a review) reduces the range of Dpp (Entchev et al., 2000). As expected, a secreted form of GFP, a protein that is unlikely to be internalized by receptor-mediated endocytosis, fills the extracellular space without forming a gradient in embryos (Pfeiffer et al., 2002) and in imaginal disks (Entchev et al., 2000). One can conclude that, independently of the mode of transport, the slope of a morphogen gradient is modulated by its rate of lysosomal degradation.

Within the framework of the planar transcytosis hypothesis, in addition to mediating degradation, endocytosis is expected to generate transport intermediates. By contrast, transport by diffusion is not expected to require endocytosis (at least at a first level of analysis; Figure 2). Therefore, experiments have been designed to ask whether transport can take place across clones of cells that are deficient in endocytosis. In a first set of experiments, the distribution of Wingless (extracellular and intracellular) was assayed in fields of cells containing *shibire*^{ts} clones (Strigini and Cohen, 2000; Figure 3B). After a 3 hr incubation at restrictive temperature, Wingless accumulates at the surface of mutant cells. Strigini and Cohen (2000) favor the interpretation that such accumulation results from continuous leakage from surrounding wild-type cells (implying diffusion across the clone). However, an alternative explanation is that *shibire*-dependent transport would slowly grind to a halt after the temperature shift (the effect of *shibire* on secretion is variable) while at the same time Wingless would become stabilized in situ and therefore be detectable 3 hr after the shift (i.e., no diffusion would be needed to account for excess Wingless at the surface of the clone). As an additional argument for diffusion, Strigini and Cohen (2000) point out that internalized Wingless is detected at the distal side of the *shibire*^{ts} clone (opposite the source), as if Wingless had diffused across endocytosis-deficient cells. However, as pointed out by Entchev et al. (2000), this could be explained by the nondirectionality of transport, which would allow transport-defective cells to be circumvented. Overall, the effects of *shibire* on the distribution of Wingless favor a diffusional mechanism of transport, but not unequivocally so.

To avoid the potential problem of circumvention of defective cells, Entchev et al. (2000) applied a so-called dynamic assay to determine whether Dpp can travel across Dynamin-deficient cells. Production of GFP-Dpp was initiated at a defined time (using the temperature sensitivity of the Gal4 system) and its distribution was then assayed at subsequent times in and around *shibire*^{ts} clones (at restrictive temperature). Oddly, within the clones, some intracellular Dpp remains (even after at least 4 hr at restrictive temperature), and unfortunately

no specific detection of extracellular Dpp was attempted. Significantly, however, in this dynamic assay a shortfall of Dpp appears behind *shibire*^{ts} clones (so-called shadows), a finding that provides seemingly compelling evidence for the planar transcytosis hypothesis (Figure 3). However, a recent paper argues on theoretical grounds that a defect in endocytosis would also lead to shadows within the context of a diffusion model (Lander et al., 2002). This is because decreased endocytosis could lead to excess receptor at the cell surface and thus prevent diffusion by trapping. Before discussing the implications of this important insight, we turn to the likely effects that receptors could have on their cognate ligands.

The Impact of Receptors

Classical cell biology points to the central role of receptors in regulating ligand endocytic trafficking. This is likely to apply to morphogens as well. For example, in wing disks, Dpp requires two receptors (encoded by *thickveins* and *saxophone*) for signal transduction (Brummel et al., 1994). Consistent with receptor-mediated endocytosis, Dpp fails to be internalized by cells lacking *thickveins* function (Entchev et al., 2000). Instead, in the absence of *thickveins*, Dpp accumulates at the cell surface (Figure 3D), possibly indicating binding to another receptor such as Saxophone or a surface proteoglycan. Wingless also utilizes multiple receptors for signal transduction (such as Frizzled, DFrizzled2, and Arrow; Bejsovec, 2000). It is likely that some or all of them are required for internalization and/or degradation, although this has not been looked at yet. In this case, we know that degradation is regulated since it is modulated both spatially and temporally in the embryonic epidermis (Dubois et al., 2001). The mode of regulation is still unknown, but it is likely to involve posttranslational modifications of intracellular residues within the relevant receptor(s) (Figure 3; Seto et al., 2002).

Despite the common theme of receptor-mediated trafficking, changing the level of receptors has been reported to affect ligand distribution in diverse, sometimes contradictory ways. Effects that have been reported include sequestering, stabilization, and trapping, as described below.

Sequestering

The first experimental evidence for the involvement of a receptor in ligand transport comes from work with Hh and its receptor, Patched (Chen and Struhl, 1996). In wing imaginal disks, Hh target genes are activated in cells located relatively near the source (e.g., Crozatier et al., 2002), consistent with the known short range of Hh in this system. Removal of *patched* in clones of cells receiving the Hh signal leads to an extension of the range across the clone as shown by the activation of Hh target genes beyond the clones, in cells that would not normally activate such targets. Conversely, overexpression of Patched leads to a reduction of the range (Chen and Struhl, 1996). Since vertebrate Patched is known to internalize Shh (Incardona et al., 2000), it is likely (although not formally proven) that in *Drosophila* wing disks, Patched captures Hh from the extracellular space and forwards it immediately to lysosomes, thus preventing it from reaching further cells (see also Denef et al., 2000).

Stabilization

A different effect is seen with DFrizzled2 and its ligand Wingless. Overexpression of DFrizzled2 leads to an overall increase of detectable Wingless protein across its normal range of action (Cadigan et al., 1998). This suggests that, under the right conditions, the receptor could contribute to ligand stabilization (maybe by protecting it from extracellular proteases). What makes a particular receptor stabilize its ligand while another receptor seems to contribute to degradation is unclear. Maybe the explanation lies in differences in the rates of endocytic trafficking, which could affect transit time in various compartments.

Trapping

Mathematical models predict that the accumulation of a large amount of free receptor at the cell surface is likely to hinder diffusion, a phenomenon called trapping (Kerszberg and Wolpert, 1998). Thus, within the context of a diffusion model, only a limited amount of receptor would be tolerated before diffusion becomes unable to contribute significantly to transport. According to Lander et al. (2002), the tolerated amount of receptor would be so low that signal transduction would be compromised unless signaling can continue after internalization. Thus, endocytosis would be an essential component of a diffusion-based model in that it would allow robust signaling under conditions of low extracellular receptor level. Indeed, numerical solution of the equations representing a diffusion model that incorporate endocytosis predicts the formation of shadows behind endocytosis-deficient cells. This is an important point because it leads to a reexamination of the most compelling argument in favor of the model of planar transcytosis. However, it does not necessarily prove the case for diffusion. For example, the diffusion model predicts that large amounts of receptor should accumulate at the surface of *shibire* mutant clones and thus lead to trapping (Lander et al., 2002), a prediction that has yet to be borne out experimentally. In the absence of further clarification, the sensible conclusion is that shadows neither prove nor disprove either model.

Are Receptors Needed for Transport?

Underlying the planar transcytosis hypothesis lies the prediction that transport would not take place across cells lacking the receptor. As mentioned above, Dpp requires Thickveins for internalization. Interestingly, Dpp accumulates at the edge of *thickveins* mutant clones, at the proximal (source) side (Figure 3; Entchev et al., 2000). This has been interpreted to mean that Thickveins is required for the transport of Dpp (in apparent contradiction with the early finding that overexpressed Thickveins decreases transport; Lecuit and Cohen, 1998). However, another interpretation is that loss of *thickveins* and the attendant loss of signaling could lead to the accumulation of a molecular species that traps Dpp. Note that such trapping would require a low off rate for association. Otherwise, within the context of any nondirectional transport (whether transcytosis or diffusion), Dpp would spread backward, down the local concentration gradient. Clearly, the role of all relevant receptors and their affinity for Dpp (alone or in heteromeric combination) needs to be investigated. The situation with Wingless is even less clear. Assessing the requirement of the signaling receptors in transport has proved

impossible so far because Wingless signaling is required for cell survival at least in wing disks (Chen and Struhl, 1999). In the case of Hh, one clear result is that its receptor Patched is dispensable for transport since the spread of Hh increases in the absence of Ptc. Therefore, if Hh is transported by planar transcytosis, it would have to be with a different receptor. One molecule that is required for Hh transport is the heparan sulfate polymerase encoded by *tout-velu* in *Drosophila* (Bellaiche et al., 1998). Therefore, a proteoglycan (a likely substrate of Tout-velu) could be involved.

In conclusion, there are many ways that a receptor could affect ligand stability and transport, and many opportunities for regulation exist, such as the rate of endocytosis and the choice between recycling and degradation. Moreover, most ligands have more than one receptor, and each could affect transport in a distinct manner. We may even have to consider the possibility that a given ligand utilizes distinct receptors for signaling and transport. For example, the proteoglycans Dally and Dally-like, two nonsignaling receptors of Wingless (Tsuda et al., 1999; Lin and Perrimon, 1999; Baeg et al., 2001), could be involved in transport (or secretion), while Frizzled and DFrizzled2 would transduce the signal. The distinct modes of association of these receptors with the cell membrane (the proteoglycans by a GPI anchor and the Frizzled receptors by multiple transmembrane domains) could lead to different trafficking behaviors (e.g., see Sabharanjak et al., 2002). More work is needed to uncover the various roles played by receptors. In particular, little information is currently available on their subcellular distribution and how it would relate to transport.

Transport Is Confined to the Plane of the Epithelium

In the context of a flat epithelium flanked by an infinite space, three-dimensional diffusion would imply that much of the ligand would be lost without ever getting a chance of reaching its receptor. (This would not apply to the spread of a signal within a mass of cells such as that of Activin through the vegetal hemisphere of *Xenopus* embryos, as so little free space would be available.) Another problem with free diffusion at the surface of an epithelium is that transport would not necessarily follow epithelial folds. This is especially relevant to leg imaginal disks of *Drosophila*, which contain many folds (Teleman et al., 2001). Therefore, within the context of epithelia, transport must strictly follow the plane of the epithelium, whether it occurs by diffusion or otherwise. How do the two models of transport cope with this requirement? Clearly, planar transcytosis fares well. However, the requirement for planar transport does not necessarily exclude passive diffusion. We can envisage various ways in which diffusion could be confined to the plane of the epithelium. For example, the basolateral interstitial space, which is bounded by the basal lamina (Figure 2), could, in principle, provide an enclosed space. However, for this to occur, the basal lamina should be impermeable to diffusible ligands and yet basally applied antibodies do reach the basal cell surface (Strigini and Cohen, 2000). An alternative possibility is that the basal lamina or the basolateral membrane harbors a meshwork of low-affinity binding site for the ligand. This would prevent the ligand from escaping from the plane of the epithelium while at the same time allowing interactions with its high-affinity receptors. Glycosaminoglycans have been suggested to be such extracellular low-affinity sites, at least for Wingless. However, the affinity has not yet been measured and the affinity of Wingless for Heparin is described as high (Reichsman et al., 1996; and thus incompatible with diffusion). Clearly, more work is needed to find out whether low-affinity sites are present in the basolateral space and whether they would allow diffusion along the plane of the epithelium.

Another means of ensuring planar transport would be for the ligand to always remain closely associated with the plasma membrane itself. Indeed, all known patterning ligands are tightly associated with the cell surface right from the time of production. For Hh, this results from direct lipid modification. Hh and its vertebrate homolog Shh are modified with cholesterol and a palmitate group (Burke et al., 1999; Chamoun et al., 2001; Ingham, 2001). Wingless is believed to remain associated to the surface of producing cells by virtue of its association with GPI-anchored proteoglycans (Baeg and Perrimon, 2000; Greco et al., 2001; Pfeiffer et al., 2002). The strength of this interaction is unknown, but is likely to depend on the strength of GPI anchorage and the affinity between Wingless and glycosaminoglycans. In addition, Wingless could also harbor lipid modifications. Although no modification is known to tether Dpp to the cell surface, the observed distribution of GFP-Dpp is compatible with tethering.

In summary, in our view, three mechanisms are compatible with the requirement that ligand should spread along the plane of the epithelium: diffusion in association with abundant and loosely arranged low-affinity sites, diffusion within the plasma membrane, and planar transcytosis. Of these, the latter two require that there be a mechanism of cell-to-cell transfer.

Cell-to-Cell Transfer

In the event that the ligand is tightly associated with cell membranes, how does it pass from one cell to another? One possibility is that specialized proteins are involved in relieving the ligand from its membrane association. A prime example here is Dispatched, a multipass transmembrane protein that has been shown to release cholesterol-modified Hh from the surface of expressing cells (Burke et al., 1999). However, Hh transport appears normal in *dispatched*-receiving cells, suggesting that this protein is not required for cell-to-cell transfer in receiving tissue. Note that Shh has been shown to form micelle-like multimers (with the cholesterol moiety in the middle) and multimeric Shh has been suggested to be the long-range form in chick limb buds (Zeng et al., 2001). It is therefore conceivable that different forms of Shh are transported by different means, although what these are is still largely unclear. In the case of other ligands, no protein is known to assist in release from the membrane.

An alternative possibility is that the ligand could cross from one cell to another by being handed directly from one receptor complex to another, like a baton in a relay race. As shown theoretically, this would be possible if

different receptor complexes with distinct affinities for the ligand existed (Kerszberg and Wolpert, 1998).

Yet another more radical possibility is that the ligand is never released from the donor cell membrane. Rather, it could be transferred to a recipient cell along with membrane in a vesicular form. Such membranous structures have been suggested to carry Wingless and have hence been named argosomes (Greco et al., 2001). As shown by Greco et al. (2001), GPI-linked GFP does transfer from cell to cell, and it is reasonable to propose that Wingless, which is likely to bind the GPI-anchored proteoglycans Dally and Dally-like, could follow the same route. Argosomes provide an interesting potential solution to the problem of cell-to-cell transfer. However, the exact cell biological mechanism remains unclear. Moreover, it is not clear whether an argosome-based transport would apply to all morphogens (Vincent and Magee, 2002).

Concluding Remarks

Which model describes best the mechanism of morphogen transport? From the start, one must be prepared for the possibility that no single model will apply. Dpp, Wingless, and Hh each have specific features and are expected to be trafficked in their own distinctive ways. As we have discussed, existing evidence favors diffusion for Wingless and transcytosis for Dpp. But for either signal, no incontrovertible argument favors one model over the other. One general argument that has been voiced against planar transcytosis is that it would be too slow to account for the observed rapid formation of morphogen gradients (Lander et al., 2002). However, in the absence of detailed knowledge about the rates of individual steps presumed to be required for transcytosis, such criticism seems premature. For example, it is argued that the off rate of the ligand-receptor interaction would be too slow to allow speedy cell-to-cell transfer. But ligand release could be an active and efficient process that has yet to be characterized. Moreover, the rate of endocytosis in transporting cells is assumed to be similar to that of cells in culture. However, developing epithelia could be specialized in fast trafficking, and intracellular trafficking of Wingless in the embryonic epidermis of *Drosophila* can be extremely rapid (Pfeiffer et al., 2002).

In the future, we expect progress to occur on several fronts. Foremost, our ability to practice rigorous cell biology will improve as new reagents become available to inhibit specific trafficking steps and hopefully measure their rates. We will also learn more about the various receptors (signaling and nonsignaling) and learn ways to interfere with specific aspects of their function. As shown recently (Lander et al., 2002), the value of models is undeniable, and it is hoped that increased cell biological knowledge will be fed into more elaborate models to predict how various parameters are likely to affect macroscopic behavior. Finally, it is important to realize that most of our current knowledge about morphogen transport comes from a limited set of experimental systems. Further progress will require an enlargement of this repertoire so that generalization could be made and that full use is made of every system's specific experimental advantages. The Zebrafish ectoderm comes to mind here. Using elegant cell transplantation experiments in a receptor mutant background, the TGFβ ligand Nodal was shown to act directly at a distance there (Chen and Schier, 2001). This experimental system in combination with gene knockdown with morpholino antisense oligonucleotides could be exploited to assess the requirement of various candidate molecules in Nodal transport.

Acknowledgments

We thank Eugenia Piddini, Derek Stemple, and James Briscoe for discussions and comments on the manuscript.

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